Immunologic Measurement of Elastin-Derived Peptides in Human Serum[1-3]

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SUMMARY Chronic obstructive pulmonary disease (COPD), a major cause of morbidity and death in the smoking population, develops insidiously over many years, and usually significant impairment of lung function has occurred before the disease is diagnosed. It is likely that destruction of the elastic fiber is a prerequisite for the development of the disease, and it is possible that immunologic identification in the serum of peptides derived from lung elastin degradation might be an effective approach to the early detection and monitoring of the disease. We prepared antibodies to peptides derived from human lung parenchymal elastin and used these antibodies in an enzyme-linked immunosorbant assay to quantitate elastin-derived peptides in the serum of 39 normal control nonsmokers, 33 smokers with normal lung function, and 40 patients with COPD. On average, statistically significant higher levels of elastin-derived peptides were found in the normal smokers and COPD patients compared to the controls. Further work with larger numbers of subjects is necessary to determine whether such a test is effective in identifying those individuals who are at risk of developing COPD.

AM REV RESPIR DIS 1983; 127:S28-S30

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and death in the smoking population. The disease usually develops insidiously over many years, and it is not until lung function has been significantly impaired that the disease can be diagnosed with certainty by radiologic and pulmonary function tests. A quantitative test that is capable of identifying the presence of lung damage at an early stage, before symptoms develop, could be useful in identifying those people who are at risk and possibly in monitoring the progression of the disease.

The connective tissue protein elastin is largely responsible for maintaining elasticity of major blood vessels and lung tissue. In experimental animal models of emphysema, the major emphasis has been on the destruction of the mature elastic fiber by selective proteases administered by aerosol or intratracheal instillation. In these systems there is a strong correlation between the production of emphysema and the cleavage of insoluble amorphous lung elastin (1-5). In humans it is also likely that destruction of the elastic fiber is a prerequisite for the development of the disease.

One possible approach to the problem of early detection and monitoring disease development is the immunologic identification of peptides derived from lung elastin degradation that may appear in the serum. As a first step in exploring the possibility, we obtained antibodies in rabbits against peptides prepared by oxalic acid or dog neutrophil elastase digestion of purified dog lung parenchymal elastin, and used these antibodies to identify and quantitate elastin-derived peptides in the serums of dogs in which emphysema had been induced by the aerosol administration of porcine pancreatic elastase (6).

In the present work we have prepared antibodies to peptides derived from human lung parenchymal elastin and used these antibodies in an enzyme linked immunosorbent assay (ELISA) to quantitate elastin-derived peptides in the serums of normal control nonsmoking individuals, smokers without evidence of disease, and individuals with COPD. The data suggest that among the three groups, the COPD patients had the highest levels whereas the normal nonsmokers had the lowest. Further investigations with larger numbers of subjects are necessary to determine whether the test will be useful in the study of human emphysema.

Methods

Purification of Elastin and Antibody Production

The amorphous component of human lung elastin was prepared by minor modification (7) of the procedure of Lansing and coworkers (8), and amino acid analysis of the preparation showed that it was highly purified elastin. Enzymatic digests of the elastin were prepared by incubation with purified human neutrophil elastase (HNE), 1 μg enzyme per mg amorphous elastin, for 24 h at 37° C in 0.05 M Tris, 0.14 M NaCl, 0.02% Na-azide pH 8.0. The reaction was terminated by the addition of 5.0 μl of 0.1 M diisopropyl fluorophosphate per ml incubation, the reaction mixture centrifuged and the supernatants used directly as antigens. The concentration of the HNE-derived elastin peptides was determined by the Lowry method (9), using bovine serum albumin as the protein standard.

New Zealand white rabbits weighing 2.5 to 4 kg were used for antibody production. Each rabbit received 0.5 mg of the solubilized elastin in a total volume of 0.5 ml of phosphate buffered saline (PBS) with an equal volume of complete Freunds adjuvant. The antigen was administered subcutaneously with multisite injections. Subsequent immunizations consisted of 0.25 mg of elastin peptides delivered weekly for at least 2 months. Seven to 10 days after the last injection, the animals were bled by venipuncture, the serums collected by centrifugation, and stored at 4° C in 0.02% NaN_3 until ready to use. Antibody titers for each animal were determined by a passive hemagglutination assay previously described (6).

Enzyme-Linked Immunosorbent Assay

Prior to the assay, the IgG fraction of both preimmune and immune serums was prepared by precipitation with 35% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 15,000 g for 15 min, suspended in PBS, pH 7.2, and dialyzed exhaustively in the cold against the same solution. The dialyzed proteins were stored at 4° C at a concentration of 1 mg/ml in PBS, containing 0.05% NaN_3. Protein concentration of the partially purified antibody was determined by measuring the absorbance at 280 nm using an extinction coefficient, $E^{1\%}_{1cm}$, of 14. All subsequent dilutions were made from the above stock solutions.

The assay was conducted essentially as described by Engvall and Pearlmann (10) with slight modifications (11). Polystyrene tubes (11 × 55 mm) were coated with HNE-human lung elastin peptides by incubation with 2.0 μg/ml peptides at 37° C for 24 h in 0.1 carbonate pH 9.6 containing 0.02% NaN_3, rinsed with PBS, and stored at 4° C

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[2] Supported by National Institutes of Health Research Grants AM-20863, AM-20553, HL-20994, and Grant 901 from the Council for Tobacco Research.

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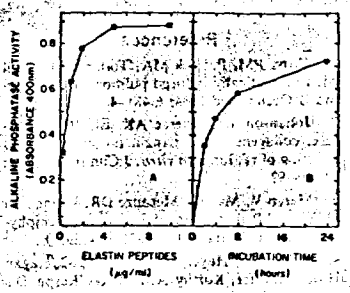

Fig. 1. (A) Binding of elastin peptides. Polystyrene tubes were coated for 24 h at 37° C with increasing concentrations of elastin peptides derived by digestion of human elastin with human neutrophil elastase, and the coated tubes incubated with 20 μg/ml specific antibody for 3 h at room temperature. The tubes were washed and ALK•PHOSPH-IgG at a 1:300 dilution was added, the tubes washed, and alkaline phosphatase activity was determined by addition of p-nitrophenyl phosphate. (B) Tubes were coated with 2 μg/ml peptide for various times at 37° C and the binding of antigen determined as in (A).

until used. Twenty micrograms of preimmune IgG and either specific antibody alone or specific antibody plus soluble antigens were preincubated at 4° C overnight in glass tubes and each solution was transferred quantitatively to precoated polystyrene tubes. The amount of specific antibody bound was quantitated by the addition of goat antirabbit IgG, to which alkaline phosphatase was covalently linked (ALK - PHOSPH-IgG) followed by reaction with its substrate, p-nitrophenyl phosphate. After 30 to 60 min incubation, the color reaction was terminated by the addition of 0.1 ml of 1.0 N NaOH and the extent of reaction measured spectrophotometrically at 400 nm. The color reaction is dependent on the amount of the bound ALK - PHOSPH-IgG which is, in turn, a measure of specific antibody uptake. A standard curve was generated using known amounts of elastin peptides: the higher the concentration of competing antigen, the greater the inhibition of color development. Subject serums were incubated with a fixed amount of specific antibody and the concentration of peptides determined from the inhibition of the reaction compared with that obtained with the standard curve. Two different amounts (25 and 150 μl) of each serum, run in triplicate, were tested.

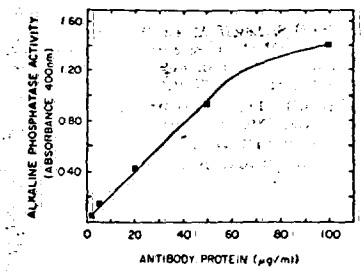

Fig. 2. Titration of elastin-specific antibody. Tubes coated with 2 μg/ml peptides were incubated for 3 h with various concentrations of elastin-specific antibody, the tubes washed, and alkaline phosphatase activity measured as in figure 1.

Patient Population Selection

A total of 109 persons were studied. They were distributed into three groups: Group I: normal nonsmokers (n = 36), Group II: normal smokers (n = 33), and Group III: COPD patients (n = 40), who were either ex-smokers or continued to smoke. Spirometry and lung volumes were determined in each by using the M-800 Autobox System produced by SRL Inc. The results were interpreted using the Intermountain Thoracic Society criteria (12). In addition, each patient filled out a detailed smoking history questionnaire, and a complete history and physical was performed on each.

Results

Development of Assay

To study the characteristics of the binding of the elastin peptides, polystyrene tubes were incubated with various concentrations of the peptides, and the extent of binding was measured using saturating amounts of specific antibody and goat antirabbit IgG. Binding was linear up to about 2 μg/ml with comparatively little additional binding at higher concentrations (figure 1A). This result suggests that maximal tight binding was reached at approximately 2 μg/ml. Binding was essentially complete by 24 h (figure 1B). Therefore, for all subsequent experiments, tubes were coated by incubation with 2 μg/ml peptides for 24 h at 37° C.

To determine the approximate concentration of specific antibody to be used in the competitive ELISA, a range of antibody concentrations was tested. A linear response was obtained up to about 60 μg/ml (figure 2). Therefore a limiting concentration of 20 μg/ml was selected for further use. A typical standard curve, illustrated in figure 3, demonstrates that the assay is useful down to about 5 ng of peptide.

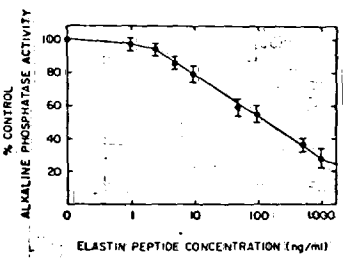

Fig. 3. Standard curve of the enzyme-linked immunosorbent assay. The competition assay was carried out as detailed in *Methods* in order to generate the standard curve. Triplicate determinations were carried out at each concentration and the bars indicate the standard deviation.

Analysis of Serum Samples

A summary of the characteristics of the three groups of subjects studied, from whom serum sample were collected, is given in table 1. As expected, the COPD patients were older, smoked longer and in greater quantity than the other 2 groups. The mean $FEV_1/FVC\%$ of 50 in Group III is consistent with moderate obstructive lung disease, whereas the $FEV_1/FVC\%$ is normal in the other two groups (> 75% is normal). The content of reactive peptides in the serum samples was quantitated using the ELISA.

The samples were assayed in a blind fashion and each was tested at a minimum of 2 dilutions, with each dilution tested in duplicate. Samples with less than 5 ng/ml were scored as zero, and only those samples that were self consistent were retained in the study.

The concentration of reactive peptides measured in the three groups is illustrated in figure 4. By inspection, one may note that in general the samples from the nonsmoking control group yielded lower values and there was a smaller variation in the values of this group compared with the control smokers with no apparent disease and the individuals with COPD. In the nonsmoking control group, a substantial fraction of the samples contained no detectable peptides with an average value of 11.9 ng/ml and the maximal value was 42 ng/ml. In the normal smokers the average value was 53.5 ng/ml with a maximal value of 190 ng/ml, and in

TABLE 1

SUBJECT POPULATION STUDIED

	N	Age	Sex	Pack/Years	Mean $FEV_1/FVC\%$
Group I Nonsmoker	36	34 ± 15	22 M 14 F	0	80 ± 5
Group II Normal smoker	33	35 ± 14	13 M 20 F	20 ± 19	78 ± 4
Group III COPD	40	57 ± 17	28 M 12 F	49 ± 31	50 ± 16

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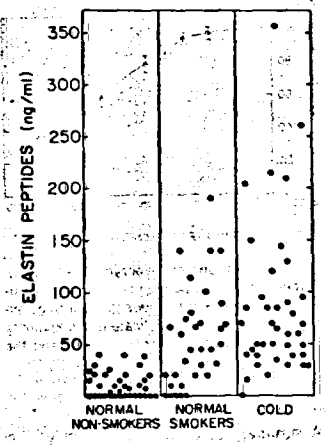

Fig. 4. Elastin peptides in human serums. Samples were assayed by the ELISA described in the text.

the COPD samples the average was 81.1 ng/ml with a maximum of 360 ng/ml.

Because of the disparity in the variance of the three groups, parametric statistics could not be employed; therefore, nonparametric statistical analyses were used. The data were first analyzed by the Kruskal-Wallis test (13). In this test all the measured values are ordered by rank and the ranks within the groups analyzed to determine whether the groups differ statistically from each other. The H value for the data illustrated in figure 4 was 46.3, a value indicating that all the groups are not identical at the $p < 0.001$ level. This test, which is a prerequisite for futher analysis, is not informative with respect to which groups differ from each other. It simply gives the probability of all groups being identical. To determine which groups differ from each other, they were analyzed by the Scheffé (14) multiple contrast test comparing pairs of groups. The results of this analysis are given in table 2. They show that both the smoking group and the COPD group have a significantly higher ($p < 0.001$) concentration of elastin-derived peptides than the control group. There also was a difference between the nonsymptomatic smokers and the COPD patients but this was significant only at the $p < 0.1$ level.

TABLE 2

ELASTIN PEPTIDES IN HUMAN SERUMS*

Group	Sample Number	$\bar{X}$ ng/ml	$\bar{R}$	$\bar{R}_i - \bar{R}_1$
1. Control	36	11.9	27.0	
2. Smokers	33	53.5	60.9	33.9†
3. COPD	40	81.1	75.4	48.4†

* Elastin-derived peptides in the sera were quantitated by the enzyme-linked immunosorbent assay described in the text.

† Significant at the $p = .001$ level. $\bar{X}$ is the average value for the elastin peptides in each of the groups. $\bar{R}$ is the average rank in each group. $\bar{R}_i - \bar{R}_1$ is the difference in average rank between either Group 2 or 3 and Group 1, the control group.

Discussion

The present studies represent the first attempt to utilize immunologic analysis of elastin-derived peptides as a means of distinguishing individuals with normal lungs from those who either presently have emphysema or are at risk of developing it. Whereas significant differences between smokers, COPD patients, and control subjects have been observed, for this test to be useful in individual cases it must be able to determine whether a given value is abnormal relative to control values in the population or whether it represents a significant change from previous measurements of a given individual. To answer these questions, and to correlate findings with physiologic measurements, chronologic studies on a larger number of individuals must be carried out in conjunction with concomitant pulmonary function tests.

Other investigators have used assays for elastin breakdown products to detect lung destruction in patients with COPD. Goldstein and colleagues (15) used a chemical assay for urinary desmosine, Harel and associates (16) used a radioimmunoassay for desmosine, and Darnule and coworkers (17) used a radioimmunoassay for elastin peptides. Although there is some disagreement regarding differences between normal smokers and normal nonsmokers, all investigators have found increased levels of elastin breakdown products in patients with COPD. It seems likely that these assays may be clinically useful tests of lung destruction after their role is fully documented in particular clinical situations.

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